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Pathogenesis of Thrombotic Microangiopathy: Insights from Animal Models

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Key Words

Thrombotic microangiopathy • Haemolytic uraemic syndrome • Thrombotic thrombocytopenic purpura • Vascular endothelial growth factor • Bevacizumab • Mouse models • Complement factor H • ADAMTS 13 • Eculizumab

Abstract

Animal models are important experimental tools for investigating the molecular mechanisms, environmental and genetic susceptibilities underlying the development of thrombotic microangiopathies. Large mammal, small animal models, knockout, transgenic and conditional knockout mouse models are available to investigate haemolytic uraemic syndrome, thrombotic thrombocytopenic purpura and vascular endothelial growth factor-associated thrombotic microangiopathy. These models have shown that it is possible to model the human conditions. However, differences in human and rodent physiology mean that caution is required when interpreting the findings. These models offer realistic prospects for identifying and testing novel therapeutic strategies in a range of thrombotic microangiopathies prior to human trials.

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Thrombotic Microangiopathies

Thrombotic microangiopathies (TMAs) are a group of conditions characterised by microvascular thrombosis leading to thrombocytopenia, haemolytic anaemia and red cell fragmentation [1]. The clinical manifestation of TMA is determined by the specificity of the process for different endothelial cell beds. This led to the historical differentiation between haemolytic uraemic syndrome (HUS) [2] and thrombotic thrombocytopenic purpura (TTP) [3]. For example, in TTP there is predominantly brain microvascular endothelial cell injury, resulting in neurological disturbance, while in HUS there is primarily glomerular endothelial cell injury resulting in acute renal impairment.

Many different aetiological predisposing factors for the development of TMA have been described. The post-diarrhoeal form of HUS (D+ HUS) is most commonly precipitated by Shiga-toxin (Stx)-producing *Escherichia coli*. Other precipitating factors for TMA include: *Streptococcus pneumoniae* infection, pregnancy, drugs (bevacizumab, mitomycin) and malignancy. However, TMAs only manifest in a small proportion of patients exposed to these environmental stimuli, suggesting additional genetic predispositions. Recent advances in molecular biology have begun to identify these factors. TTP has been shown to be predisposed to by a genetic deficiency of

Table 1. Toxin models of TMAs

Model	Trigger	Pathology	Ref.
<i>Shiga toxins</i>			
Mouse	oral <i>E. coli</i> O157:H7; Sm	renal cortical tubular necrosis	Wadolkoski et al. (1990)
Mouse	IV/IP Stx1, Stx2	necrosis of tubular cells	Tesh et al. (1993)
Mouse	IG <i>E. coli</i> O157:H7	tubular necrosis	Karpman et al. (1997)
Mouse	SQ IP LPS + IV Stx2	tubular necrosis	Palermo et al. (1999, 2000)
Mouse	IV Stx2	widespread mesangial hypercellularity, crescent formation	Dran et al. (2002)
Mouse	IV Stx2 + L-NAME	widespread mesangial hypercellularity, crescent formation, glomerular thrombosis, tubular necrosis	Dran et al. (2002)
Mouse	IV Stx1, Stx2	necrosis of tubular cells	Rutjes et al. (2002)
Mouse	SQ IP LPS + Stx2	mild glomerular endothelial damage	Ikeda et al. (2004)
Mouse	IP LPS + Stx2	thrombocytopenia, haemolytic anaemia, renal failure, fibrin thrombi	Keepers et al. (2006, 2007)
Mouse	IP Stx2	haemolysis, renal failure, glomerular fibrin deposition	Sauter et al. (2008)
Mouse	oral enterohemorrhagic <i>E. coli</i> germ-free mice	renal tubular necrosis, glomerular capillary RBC sludging and occasional fibrin thrombi	Eaton et al. (2008)
Rat	IV Stx2	acute tubular injury	Zhao et al. (2002)
Rat	IP <i>E. coli</i> Stx2 supernatant	tubular + glomerular necrosis, glomerular fibrin thrombi	Zotta et al. (2008)
Ferret	oral <i>E. coli</i> O157:H7; Sm	some develop glomerular fibrin thrombi, occasionally thrombocytopenia	Woods et al. (2002)
Dutch belted rabbits	oral <i>E. coli</i> O153	glomerular fibrin thrombi + tubular necrosis	Garcia et al. (2002)
Gnotobiotic piglets	oral <i>E. coli</i> O157:H7	glomerular TMA, no renal failure, no thrombocytopenia	Gunzer et al. (2002)
Greyhounds	oral <i>E. coli</i> O157:H7	glomerular TMA	Hertzke et al. (1995)
Baboon	IV Stx1, Stx2	renal TMA	Taylor et al. (1999)
		thrombocytopenia, MAHA, renal failure	Siegler et al. (2003)
<i>Endothelial toxins</i>			
Mouse	IV/IP ricin ± LPS	thrombocytopenia, haemolytic anaemia, renal failure, glomerular fibrin thrombi	Taylor et al. (1999)
Mouse	transplantation of hybridoma clone (anti-platelet Ab)	thrombocytopenia, microthrombi in small vessels of lung	Hashimoto et al. (2000)
Mouse	SQ RAP Con A + anti-Con A Ab	glomerular + peritubular microvascular thrombosis + tubular necrosis	Hohenstein et al. (2008)
Rat	mitomycin perfusion of kidney	renal TMA	Cattell et al. (1985)
Rat	RAP anti-GEC Ab	renal TMA	Nangaku et al. (1997)
Rat	IV anti-EC Ab	thrombocytopenia, tubular injury, occasional glomerular intracapillary thrombi	Ren et al. (2002)
<i>Other</i>			
Dog/pig	botrocetin	platelet microthrombi in lungs and spleen	Sanders et al. (1995)
Rats	botrocetin	platelet microthrombi in lungs and spleen	Sanders et al. (1988)

Sm = Streptomycin pre-treated; IV = intravenous; IP = intraperitoneal; LPS = lipopolysaccharide; SQ = sequential administration; IG = intragastric; GEC = glomerular endothelial cell; EC = endothelial cell; RAP = renal artery perfusion; RBC = red blood cell; TMA = thrombotic microangiopathy.

ADAMTS13 (the rare Upshaw-Schulman syndrome) or more commonly from production of inhibitory anti-ADAMTS13 antibodies [3]. By contrast, the non-diarhoeal form of HUS, atypical HUS (aHUS), is predisposed to by mutations in, or antibodies against, complement regulatory proteins [2].

Discovery of these genetic predispositions has allowed generation of specific knockout and transgenic mouse models to add to existing Stx models of TMA. These are now yielding significant advances in the understanding of disease pathogenesis and provide models to screen potential therapeutic agents. The strengths and possible limitations of this experimental approach are reviewed here.

Animal Models of Thrombotic Microangiopathies (tables 1, 2)

Initial attempts to generate animal models of thrombotic microangiopathies focused on oral administration of Stx-producing *E. coli* or parenteral administration of Stx.

Non-Rodent Models of D+ HUS

Greyhound – Shiga Toxin

‘Alabama rot’ is a naturally occurring model of D+ HUS which was first recognised at a greyhound race track in Alabama. Racing greyhounds are fed raw meat from rendering plants which commonly contains pathogenic

Table 2. Genetically modified models of TMAs

Model	Trigger	Pathology	Ref.
<i>Complement k/o</i>			
Norwegian Yorkshire pig	spontaneous	MPGN	Hegasy et al. (2002)
Mouse CFH ^{-/-}	spontaneous	MPGN	Pickering et al. (2002)
Mouse CFH ^{+/-}	IP Stx2 + LPS	no TMA, no difference in tubular injury compared to controls	Paixao-Cavalcante et al. (2009)
Mouse CFH ^{-/-} Δ16–20	spontaneous	aHUS	Pickering et al. (2007)
Mouse C5 ^{-/-} CFH ^{-/-} Δ16–20	spontaneous	no aHUS	Goicoechea de Jorge et al. (2008)
Mouse CFI ^{-/-}	spontaneous	mesangial C3 deposits with nodular expansion	Rose et al. (2008)
<i>ADAMTS 13 k/o</i>			
Mouse ADAMTS 13 ^{-/-} (129/Sv)	N/A	none	Banno et al. (2006)
Mouse ADAMTS 13 ^{-/-} (C57BL/6;129x1/Sv)	N/A	none	Motto et al. (2005)
Mouse ADAMTS 13 ^{-/-} (CASA/Rk)	spontaneous	TTP like lesions (in some)	Motto et al. (2005)
Mouse ADAMTS 13 ^{-/-} (CASA/Rk)	IV Shiga toxin	TTP like lesions (in most)	Motto et al. (2005)
<i>VEGF k/o</i>			
Mouse VEGFA ^{-/-}	spontaneous	major vascular defects	Carmeliet et al. (1996)
Mouse podocyte VEGFA ^{-/-}	spontaneous	perinatal death, grossly abnormal glomeruli	Eremina et al. (2003)
Mouse conditional podocyte VEGFA ^{-/-}	spontaneous	TMA	Eremina et al. (2008)
<i>Renin angiotensin TG</i>			
R ⁺ /A ⁺	high salt diet/L-NAME	glomerular fibrin thrombi, mesangiolysis	Sethi et al. (2006)

E. coli. These dogs develop bloody diarrhoea, skin ulcers and renal failure. The renal pathology is of an extensive glomerular TMA. Preliminary experiments using this model demonstrated that treatment with Lepirudin, a recombinant hirudin anticoagulant that binds thrombin, prevented the lethal effects of Stx2.

Baboon – Shiga Toxin

Siegler et al. [4] developed a baboon model of Stx-mediated HUS. Animals given IV Stx1 or Stx2 developed progressive thrombocytopenia, haemolytic anaemia and renal failure. Glomerular thrombotic microangiopathy was found at necropsy. This model demonstrated that Stx2 is a more potent initiator of D+ HUS than Stx1.

Rodent Models of D+ HUS

Small Animal Models – Shiga Toxin

Thus, canine and baboon models of HUS mimic the human disease; however, they are impractical and costly. Small animal models of Stx HUS have proved a poor homologue of human disease, with most developing tubular damage without glomerular thrombosis (table 1). This is felt to relate to interspecies variability in the expression of the Stx receptor, Gb3, which is absent in mouse glomeruli. However, murine renal tubules do express Gb3, which provides an explanation for the pattern of disease in mice exposed to Stx.

Keepers et al. [5] recently demonstrated that intraperitoneal co-injection of purified Stx2 plus lipopolysaccharide in mice resulted in the classical triad of thrombocytopenia, haemolytic anaemia and renal failure seen in human HUS. However, it has been suggested that this glomerular pathology could follow primary tubular damage [6].

Endothelial Cell Toxin Model of TMA

Mitomycin C, an alkylating chemotherapy agent, is thought to cause renal thrombotic microangiopathy by a direct toxic effect on endothelium. When mitomycin was perfused into kidneys of rats, a pathological picture resembling HUS resulted, with fibrin deposition and endothelial cell proliferation.

Antibody-Mediated Models of TMA

Nangaku et al. [7] generated a rat model of TMA that histologically and clinically resembles HUS by selective unilateral renal artery perfusion of antibodies against glomerular endothelial cells. This injury was prevented by using rats that had undergone complement depletion using cobra venom factor or were genetically C6-deficient, suggesting a role for membrane attack complex formation in the pathogenesis. Additional studies using this model also suggested a role for C5b-9-mediated endothelial cell apoptosis and showed protective effects of VEGF.

A further rat model of TMA with renal disease was developed by Ren et al. [8], using a complement-fixing antibody to endothelial cells. EC injury was prevented by complement depletion and worsened by blockade of Crry. Hashimoto et al. [9] generated a hybridoma clone in lupus-prone mice (MRL/*lpr*) which, when transplanted into syngeneic non-autoimmune mice, caused microvascular intraluminal platelet aggregation, thrombocytopenia and anaemia. This pathogenic autoantibody (anti-gp70) specifically precipitated a platelet protein with an approximate relative molecular mass of 40 kDa. These studies replicate human studies that have demonstrated the presence in patients' plasma of IgG antibodies reactive with a platelet and/or endothelial cell antigen, especially CD36 in HUS and TTP.

Specific Pathophysiological Models

Complement Mouse Models of aHUS

Mutations in the complement regulatory proteins have been shown to predispose to aHUS [2]. This has provided alternative targets for the generation of mouse models of aHUS. Mutations in the complement regulator factor H have been linked to two different human renal diseases: membranoproliferative glomerulonephritis (MPGN) and aHUS. Although both are associated with complete factor H deficiency, aHUS is more commonly associated with heterozygous mutations in the C-terminal of *CFH* resulting in normal plasma levels of CFH.

To investigate the role of factor H in disease, Pickering et al. [10] developed a mouse *CFH* knockout. This *CFH*^{-/-} mouse, which demonstrated uncontrolled turnover of the alternative pathway with very low C3 levels, developed MPGN, not aHUS. A spontaneously arising *CFH*-deficient strain of pig has also been shown to develop MPGN, not aHUS.

In order to better mimic the situation seen in aHUS, Pickering et al. [11] generated a transgenic mouse on the background of the *CFH*^{-/-} mouse which lacked the 5 C-terminal CCPs of *CFH* (*CFH*^{-/-}Δ16–20). It is this region, shown to be important for cell-surface binding [12], which contains 80% of the factor H mutations reported to cause human aHUS.

This *CFH*^{-/-}Δ16–20 mouse, which had higher plasma C3 levels compared to the *CFH*^{-/-} mouse, developed aHUS, not MPGN. Thus, this mouse model provides the first in vivo evidence that the *CFH* mutations seen in aHUS impair endothelial cell surface recognition, resulting in local complement dysregulation, while controlling

the alternative pathway in plasma (reviewed in Pickering et al. [13]). In these mice only the homozygous transgenic mice developed HUS, whereas in humans a heterozygous mutation is sufficient to predispose to aHUS. This may reflect the differences in complement regulation between the mouse and the human. Alternatively, it may mimic the human situation in which individuals are predisposed to aHUS by a *CFH* mutation, but require an additional trigger of complement activation (e.g. infection, drugs) for disease to manifest. Further investigation of the heterozygote mouse response to such stimuli will be instructive.

De Jorge et al. [14] have also crossed the *CFH*^{-/-}Δ16–20 with a C5-deficient mouse to investigate the role of C5 activation in the pathogenesis of aHUS. These *C5*^{-/-}*CFH*^{-/-}Δ16–20 mice, did not develop aHUS, suggesting a critical role downstream of C3b generation in aHUS and providing a rationale for use of C5 inhibition (e.g. eculizumab) in the treatment of aHUS.

Heterozygous mutations in complement factor I have also been shown to predispose to aHUS. The factor I knockout mouse (*CFI*^{-/-}) shows uncontrolled alternative pathway activation; however, they do not develop aHUS, the pathological picture is of mesangial C3 deposits with nodular expansion.

Although mutations in membrane cofactor protein (CD46; *MCP*) are associated with aHUS, the differences between human and murine complement systems preclude an adequate mouse model. While CD46 is highly expressed on most human tissues including glomerular endothelium, in rodents CD46 is limited to the testes. In place of CD46, rodents express the rodent-specific complement regulatory protein, Crry. Crry has decay-accelerating and co-factor activity. Although embryonically lethal, the *Crry* knockout mouse (*Crry*^{-/-}) can be rescued on a C3-deficient mouse (*Crry*^{-/-}*C3*^{-/-}). When kidneys from *Crry*^{-/-}*C3*^{-/-} mice are transplanted into mice with a functioning complement system, the kidneys fail due to uncontrolled complement activation, principally in the tubulointerstitium. Although not the pathological picture of HUS, this confirms the necessity of Crry in the mouse kidney and highlights the differences between human and murine complement systems.

ADAMTS13 Knockout Models of TTP

Thrombotic thrombocytopenic purpura has recently been shown to be a disorder of von Willebrand Factor (vWF) regulation [3]. In response to vascular injury, stored vWF is released from endothelial cells as ultra large multimers (UL-vWF). However, some UL-vWF remain associated with the endothelial surface, providing

binding sites for platelets and possibly other blood components such as leukocytes. The presence of UL-vWF may lead to spontaneous platelet aggregation in the circulation or on the endothelial cell surface if it is not rapidly processed by the metalloprotease ADAMTS13. The discovery of ADAMTS13 as crucial to the pathogenesis of TTP led to two groups generating knockout mice in an attempt to produce an animal model of TTP resembling the rare human Upshaw-Schulman syndrome.

Motto et al. [15] produced an ADAMTS13 knockout in two strains of mice. On the initial background (mixed genetic background C57BL/6 and 129×1/Sv), which was subsequently shown to express a truncated form of ADAMTS 13, there was complete loss of ADAMTS13 activity. vWF-mediated interactions between platelets and vascular endothelium were prolonged compared to wild type. Despite this, there was no evidence of a thrombotic microangiopathy and no difference in survival compared to wild type. When this ADAMTS13^{-/-} mouse was crossed onto the genetically distant CASA/Rk mice (which express full length ADAMTS13), a number of the mice developed spontaneous thrombocytopenia with increased mortality. These mice had the vWF-rich and fibrin-poor hyaline thrombi characteristic of TTP in the small vessels of multiple organs, including the kidney. When these susceptible CASA/Rk ADAMTS13^{-/-} mice were exposed to IV Stx, most developed a picture consistent with TTP that was not seen in the CASA/Rk ADAMTS13^{+/+} mice.

As the CASA/Rk mice have higher plasma vWF levels than the mixed genetic background mouse, it had been proposed that this accounted for the difference in phenotype between these two models. However, early generations of the cross between these two mice produced a random inheritance of vWF-regulatory factors leading to a heterogeneous population of knockouts with a wide range of vWF levels. In these mice, no correlation was seen between plasma vWF and the degree of Stx-induced thrombocytopenia or mortality. This suggests that the non-susceptible strain may contain other genetic differences that may protect against TMA.

Banno et al. [16] subsequently produced an ADAMTS13 knockout on pure strain 129/Sv mice, but these mice failed to show any evidence of thrombotic microangiopathy. The ADAMTS13^{-/-} mice did, however, show a UL-VWF multimer pattern, similar to that seen in TTP, compared to the ADAMTS13^{+/+} which demonstrated a normal multimer pattern. When these ADAMTS13^{-/-} mice were challenged with the platelet and endothelial agonists, collagen and epinephrine, they developed a se-

vere thrombocytopenia compared to controls. However, mortality between the two groups was no different.

Thus, these mice suggest that, in addition to ADAMTS13 deficiency, additional environmental and genetic triggers are required for TTP to manifest.

In addition to the ADAMTS13 knockout mice models of familial TTP, the ADAMTS 13^{+/+} (on the non-TTP susceptible mouse strain) was injected with polyclonal rabbit antihuman ADAMTS13 antiserum in an effort to replicate acquired TTP. These mice demonstrated significantly prolonged vWF-mediated platelet-endothelial interactions, similar to the untreated ADAMTS13^{-/-} mice not receiving antiserum [17], but did not develop TTP.

VEGF Models of TMA

Vascular endothelial growth factor (VEGF) is one of the most important endothelial cell growth factors. In addition to its role in induction and maintenance of normal vascular endothelium it is markedly upregulated in many human tumours. Bevacizumab is a humanised monoclonal antibody against VEGF which, as part of chemotherapy regimens, improves survival rates by preventing angiogenesis. A rare complication of this therapy has been the development of renal TMA. In the kidney, VEGF is constitutively expressed in the glomerular podocytes. To examine the role of VEGF in TMAs, various mouse models of VEGF-A deficiency have been created.

VEGF-A^{-/-} and VEGF-A^{+/-} mice are embryonic lethal due to major vascular defects. To examine the role of VEGF in the kidney, a podocyte-specific deletion of VEGF was generated in mice. Podocyte-specific homozygotes died perinatally with kidney failure and grossly abnormal glomeruli which lacked mature endothelial cells. Mice with podocyte-specific heterozygosity for VEGF developed endotheliosis and bloodless glomeruli, the renal lesion seen in pre-eclampsia, which progressed to nephrotic syndrome and then renal failure. In contrast, overexpression of the 164 isoform of VEGF-A in podocytes led to a collapsing glomerulopathy, which is the renal lesion seen in HIV-associated nephropathy [18]. Thus, VEGF is critical for the normal development of the glomerulus.

To test the role of VEGF inhibition in mature kidney, Eremina et al. [19] used conditional gene targeting to delete VEGF from the renal podocytes in adult mice. These mice developed typical features of thrombotic microangiopathy with intracapillary thrombi on electron microscopy mirroring the thrombotic microangiopathy seen in patients treated with bevacizumab. This suggests a protective role of VEGF in maintaining glomerular vascular

integrity and preventing the endothelial cell damage which may result in TMA.

Renal Thrombotic Microangiopathy in a Genetic Hypertension Mouse Model

Malignant hypertension in humans can result in TMA. Transgenic mice that are hypertensive because of overexpression of the human renin (R^+ mice) and angiotensin (A^+ mice) genes develop renal thrombotic microangiopathy when placed on a high salt diet and/or nitric oxide synthase inhibitor, L-NAME, is added to drinking water. This model may lead to renal TMA by (a) hypertensive endothelial cell injury, (b) inhibition of nitric oxide synthase or (c) direct effect of high salt on renal blood vessels.

What Are the Dangers of Extrapolation from Mice Models of TMA to Humans?

The simple transfer of knowledge from mice to humans is highly illusive. Because there are so many similarities, there is a tendency to ignore differences and make the assumption that what is true in mice is true in humans. The genomes of mice and humans diverged approximately 65–75 million years ago. Mice and humans differ significantly in terms of size, lifespan and immunological systems [20]. For example, of potential significance to the pathophysiology of TMAs are differences in the balance of leukocyte subsets, chemokine and cytokine receptor expression, inducible nitric oxide synthase and complement regulatory proteins. Additionally, mice are housed in pathogen-free areas, and do not usually have other comorbid conditions. Mice are frequently inbred and the phenotype obtained can be dependent on the strain used, as in the ADAMTS13 $^{-/-}$ mouse models. One concern regarding the use of knockout mice is that the development of the immune system, in the absence of

a specific protein, may result in the generation of confounding and unanticipated compensatory mechanisms. The use of transgenic mice may to some extent address these issues. This seems to be supported by the evidence from the podocyte conditional knockout of VEGF and the CFH $^{-/-}$ Δ16–20 mouse, which closely replicated the human conditions they attempted to model. Finally, despite the hope that animal models will allow rapid identification of treatments for TMAs, there are many examples in the literature where therapeutic strategies developed in mouse models have not translated into successful human trials, e.g. blockade of TNF α for treatment of sepsis.

Conclusions

Using the wide range of genetic and environmental triggers of TMAs a large number of animal models have been developed. The initial small animal models of Stx-HUS were a poor imitation of the human disease while the large animal models are impractical for most laboratories to use. However, the recent introduction of genetically modified mice have probed specific pathways known to be perturbed in humans and this has provided better models of human disease. These mice may serve as a useful platform for testing novel therapeutic strategies in these important conditions.

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